Southern Transfer

The techniques described on the previous pages yield a map that displays the order of restriction sites and the sizes of restriction fragments. Localization of particular sequences of DNA within these fragments is usually accomplished by the transfer technique described by Southern (1975). DNA fragments that have been separated according to size by electrophoresis through an agarose gel are denatured, transferred to a nitrocellulose filter, and immobilized. The relative positions of the DNA fragments in the gel are preserved during their transfer to the filter. The DNA attached to the filter is then hybridized to ^{32}P-labeled DNA or RNA, and autoradiography is used to locate the position of any bands complementary to the radioactive probe. This technique can be used not only to locate specific sequences in cloned DNA, but also to identify sequences within digests of total eukaryotic DNA (Botchan et al. 1976; Jeffreys and Flavell 1977). The techniques described on the following pages are applicable to both genomic and cloned DNA, with only minor modifications.

TRANSFER OF DNA FROM AGAROSE GELS TO NITROCELLULOSE PAPER

1. After electrophoresis is completed, stain the DNA with ethidium bromide and photograph the gel. It is sometimes useful to place a ruler alongside the gel so that the distance that any given band of DNA has migrated can be read directly from the photographic image.

   Half a microgram of the DNA of a recombinant λ bacteriophage or 0.2 μg of a recombinant plasmid DNA is more than sufficient to allow inserted DNA sequences to be easily detected by Southern hybridization. However, 10 μg of total mammalian DNA (haploid genome = 3×10^9 bp) must be applied to a single gel-slot in order to be able to detect sequences that occur only at the single-copy level.

2. Transfer the gel to a glass baking dish and trim away any unused areas of the gel with a razor blade.

3. Denature the DNA by soaking the gel in several volumes of 1.5 M NaCl and 0.5 M NaOH for 1 hour at room temperature with constant stirring or shaking.

   Note. After electrophoresis, some investigators prefer to hydrolyze the DNA partially by acid depurination (by soaking the gel twice for 15 minutes in 0.25 M HCl at room temperature) prior to alkali denaturation (Wahl et al. 1979). This acid-induced cleavage aids in the transfer of large DNA fragments. However, it is important not to let the hydrolysis reaction proceed too far; otherwise the DNA is cleaved into fragments that are too short to bind efficiently to the filter (<300 bp). In most cases, sufficient breakage occurs during the time that the ethidium-bromide-stained DNA is exposed to UV irradiation to allow efficient transfer of DNA up to 20 kb in length.

4. Neutralize the gel by soaking in several volumes of a solution of 1 M Tris · Cl (pH 8.0) and 1.5 M NaCl for 1 hour at room temperature with constant shaking or stirring.

5. Wrap a piece of Whatman 3MM paper around a piece of plexiglass or a stack of glass plates. Place the wrapped support inside a large baking dish. The support should be longer and wider than the gel. Fill the dish with 10× SSC (see page 447) almost to the top of the support and smooth out all air bubbles in the 3MM paper with a smooth glass rod.

   Note. 20× SSPE can also be used as the transfer buffer.

6. Invert the gel so that its original underside is now uppermost. Place the gel on the damp 3MM paper. Make sure there are no air bubbles between the 3MM paper and the gel.

7. Using a fresh scalpel or a paper cutter, cut a piece of nitrocellulose filter (Schleicher & Schuell BA 85 or Millipore HAHY) about 1–2 mm larger than the gel in both dimensions. Use gloves and Millipore forceps to handle the nitrocellulose.

8. Float the nitrocellulose filter on the surface of a solution of 2× SSC until it wets completely from beneath. Then immerse the filter in the 2× SSC for 2–3 minutes.
   The rate at which different batches of nitrocellulose wet varies enormously. If the filter is not saturated after several minutes, it should be replaced with a new piece of nitrocellulose since the transfer of DNA to unevenly wet nitrocellulose is unreliable.

   Note. Nitrocellulose that has been touched by greasy hands will never wet!

9. Place the wet nitrocellulose filter on top of the gel, so that one edge extends just over the line of slots at the top of the gel. Be careful to remove all air bubbles that are trapped between the gel and the filter.

10. Wet two pieces of Whatman 3MM paper, cut to exactly the same size as the gel, in 2× SSC and place them on top of the nitrocellulose filter. Again remove all air bubbles.

11. Cut a stack of paper towels (5–8 cm high) just smaller than the 3MM paper. Place the towels on the 3MM paper. Put a glass plate on top of the stack and weigh it down with a 500-g weight (see Fig. 11.1). The objective is to set up a flow of liquid from the reservoir through the gel and the nitrocellulose paper, so that DNA fragments are eluted from the gel and are deposited onto the nitrocellulose paper. To prevent short circuiting of fluid between the paper towels and the 3MM paper under the gel, many workers surround the gel with a water-tight border of Saran Wrap.

12. Allow transfer of DNA to proceed for about 12–24 hours. As the towels become wet, they should be replaced.
    The rate of transfer of DNA depends on the size of the DNA fragment and the porosity of the gel. Small fragments of DNA ($<$ 1 kb) transfer from an 0.8% agarose gel within an hour or 2 while transfer of DNA greater than 15 kb takes 15 hours or more.

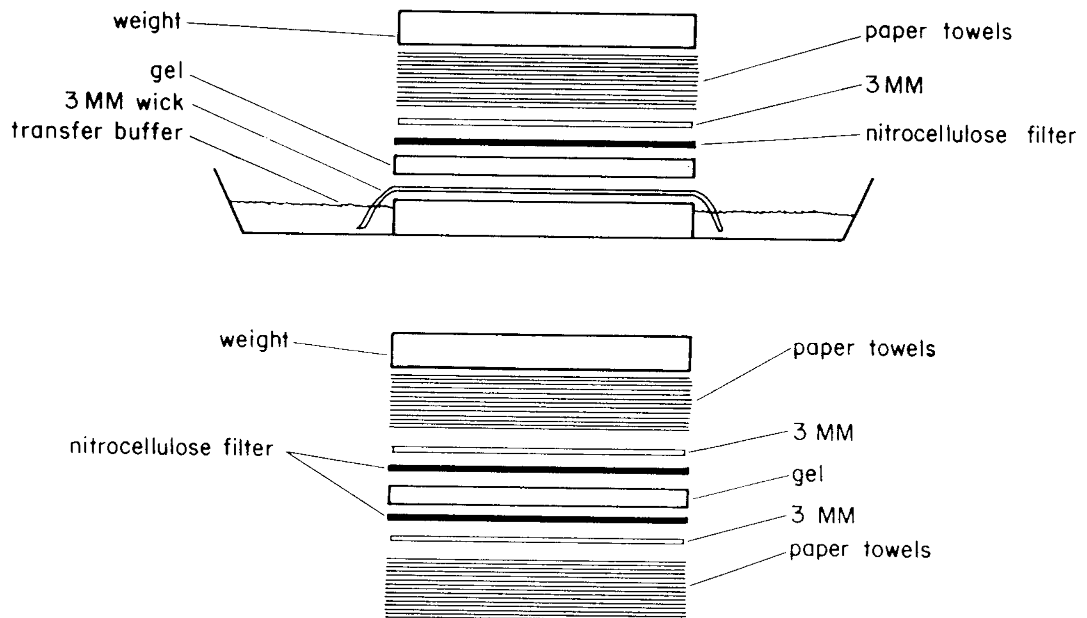

Figure 11.1

Methods for transfer of DNA from agarose gels to nitrocellulose filter paper. *(Top)* The most common system for transfer of DNA (see text for explanation). *(Bottom)* A system for preparing duplicate nitrocellulose filters from a single gel; the transfer buffer is supplied only by the liquid in the agarose gel itself.

13. Remove the towels and the 3MM filters above the gel. Turn over the dehydrated gel and filter and lay them, gel side up, on a dry sheet of 3MM paper. Mark the positions of the gel slots on the filter with a very soft pencil or a ball-point pen.

14. Peel off and discard the gel. Soak the filter in 6× SSC at room temperature for 5 minutes.

15. Allow excess fluid to drain from the filter and set the filter to dry at room temperature on a sheet of 3MM paper.

16. Place the dried filter between two sheets of 3MM paper. Bake for 2 hours at 80°C under vacuum.

    If the filter is not to be used immediately in hybridization experiments, it should be stored at room temperature under vacuum between sheets of 3MM paper.

Notes

i. The procedure given above and illustrated in Figure 11.1 (top) is the most widely used of many different methods that are employed to transfer DNA from gels to filters. Of the alternative set-ups, perhaps the best is that shown in Figure 11.1 (bottom) in which DNA is transferred from a single gel to two nitrocellulose filters simultaneously. After denaturation and neutralization (steps 3 and 4), the gel should be soaked for 1 hour in 20× SSC or 20× SSPE. Transfer of small DNAs (<5 kb) then occurs extremely rapidly and is essentially complete in 3–4 hours. However, the only source of transfer buffer is the liquid trapped in the gel itself, and transfer of high-molecular-weight DNA fragments (>10 kb) is therefore somewhat inefficient.

ii. Marker DNAs that will hybridize to the radioactive probe can serve simultaneously to orient the filter and to provide size markers directly on the autoradiograph. The amount of DNA in the marker lane that will hybridize to the probe should be equal to or only slightly more than the amount of probe-specific DNA in the sample lanes.

HYBRIDIZATION OF SOUTHERN FILTERS

1. Float the baked filter on the surface of 6× SSC until it wets from beneath. Immerse the filter in the 6× SSC for 2 minutes.

2. Slip the wet filter into a heat-sealable plastic bag (e.g., Sears' Seal-n-Save).

3. Add 0.2 ml of prehybridization fluid warmed to 68°C for each square centimeter of nitrocellulose filter.

   Prehybridization fluid

   6× SSC
   0.5% SDS
   5× Denhardt's solution (see page 448)
   100 μg/ml denatured, salmon sperm DNA (see page 327)

4. Squeeze as much air as possible from the bag. Seal the open end of the bag with the heat sealer. Incubate the bag for 2–4 hours submerged in a water bath at 68°C.

   Often, small bubbles of air form on the surface of the filter as the temperature of the prehybridization solution rises to 68°C. It is important that these bubbles be removed by occasionally agitating the fluid in the bag; otherwise the components of the prehybridization fluid will not be able to coat the filter evenly.

5. Remove the bag from the water bath. Open the bag by cutting off one corner with scissors. Squeeze out as much prehybridization solution as possible.

6. Using a pasteur pipette, add the hybridization solution to the bag. Use just enough solution to keep the filter wet (50 μl/cm^2 of filter).

   Hybridization solution

   6× SSC
   0.01 M EDTA
   ^{32}P-labeled denatured probe DNA
   5× Denhardt's solution
   0.5% SDS
   100 μg/ml denatured, salmon sperm DNA

   Typical hybridization conditions for Southern filters are given in Table 11.1.

7. Squeeze as much air as possible from the bag. Seal the cut edge with the heat sealer so that as few air bubbles as possible are trapped in the bag.

TABLE 11.1 HYBRIDIZATION CONDITIONS FOR SOUTHERN FILTERS

DNA on filter	Sp. act. of probe DNA (cpm/μg)	Amount of probe added	Time of hybridization (hr)
Fragments of cloned DNA (~ 100 ng/fragment)	10^7	10^5–10^6 cpm (0.01–0.1 μg)	3–4
Total eukaryotic DNA (10 μg)	10^8	1×10^7 cpm –5×10^7 (0.1–0.5 μg)	12–16

8. Incubate the bag submerged in a water bath at 68°C for the required hybridization period.

9. Remove the bag from the water bath and quickly cut along the length of three sides. Using gloves, remove the filter and immediately submerge it in a tray containing a solution of 2× SSC and 0.5% SDS at room temperature.

   Note. Do not allow the filter to dry out at any stage during the washing procedure.

10. After 5 minutes, transfer the filter to a fresh tray containing a solution of 2× SSC and 0.1% SDS and incubate for 15 minutes at room temperature with occasional gentle agitation.

11. Transfer the filter to a flat-bottomed plastic box containing a solution of 0.1× SSC and 0.5% SDS. Incubate at 68°C for 2 hours with gentle agitation. Change the buffer and continue incubating for a further 30 minutes.

    Note. If the homology between the probe and the DNA bound to the filter is inexact, the washing should be carried out under less stringent conditions. In general, washing should be carried out at $T_m - 12°C$.

    The following relationships are useful:

    a. $T_m = 69.3 + 0.41 \cdot (G + C)\% - 650/L$.
       L equals the average length of the probe in nucleotides (Marmur and Doty 1962; Wetmer and Davidson 1968).

    b. The T_m of a duplex DNA decreases by 1°C with every increase of 1% in the number of mismatched base pairs (Bonner et al. 1973).

c. $(T_m)\mu_2 - (T_m)\mu_1 = 18.5 \log_{10} \frac{\mu_2}{\mu_1}$

where μ_1 and μ_2 are the ionic strengths of two solutions (Dove and Davidson 1962).

12. Dry the filter at room temperature on a sheet of Whatman 3MM paper.

13. Wrap the filter in Saran Wrap and apply to X-ray film to obtain an autoradiographic image (see page 470).

Notes

Hybridization may also be carried out in:

a. flat-bottomed plastic boxes.

b. buffers containing formamide. Each increase of 1% in the formamide concentration lowers the T_m of a DNA duplex by 0.7°C (McConaughy et al. 1969; Casey and Davidson 1977).